

Hypoxia, tumor oxygen dynamics and their assessment

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There is increasing evidence for the importance of tumor oxygenation in development, progression, and response to therapy. Consequently, many techniques have been developed to assess tumor oxygenation, as reviewed extensively (1-7). Methods may provide a qualitative impression of oxygenation status or rigorous quantitation. Techniques vary in spatial and temporal resolution and the ability to assess dynamic changes. Some exploit endogenous molecules or physical characteristics, while many apply reporter molecules to interrogate oxygen tension (pO_2). This tutorial will focus on magnetic resonance approaches, but place them in the context of competing modalities.

It has long been appreciated that hypoxic tumor cells are relatively resistant to radiotherapy. Indeed, a three fold increase in radio resistance may occur when cells are irradiated under hypoxic conditions compared with $pO_2 > 15$ torr for a single radiation dose. However, recent modeling indicates that the proportion of cells in the range 0 - 20 torr may be most significant in terms of surviving a course of fractionated radiotherapy (8). Increasingly, there is evidence that hypoxia also influences such critical characteristics as angiogenesis, tumor invasion and metastasis (4, 9). Thus, the ability to measure pO_2 non-invasively, and repeatedly, with respect to acute or chronic interventions becomes increasingly important. Patients could be stratified according to baseline hypoxia to receive adjuvant interventions designed to modulate pO_2 , or more intense therapy as facilitated by IMRT (Intensity Modulated Radiation Therapy). Tumors, which do not respond to interventions, may be ideal candidates for hypoxia selective cytotoxins (e.g., tirapazamine). Noting that any therapy and intervention may have side effects or simply add to clinical costs, it is vital that efficacy be established and therapy be optimized for an individual patient. Whether initially hypoxic regions of a tumor can be modified to become better oxygenated has long been considered a key to improving outcome of irradiation. However, many attempts to improve therapeutic outcome by manipulation of tumor oxygenation have shown only modest success in the clinic (10) and it is thought that lack of success may have resulted from inability to identify those patients, who would benefit from adjuvant interventions. While pO_2 determinations could be of great clinical value, they are also vital to many laboratory investigations of new drugs and studies of tumor development.

Many reports have now shown that tumors are highly heterogeneous and have extensive hypoxia. Furthermore, strong correlations have been shown in cervix and head and neck tumors between median pO_2 or hypoxic fraction and survival or disease free survival using the Eppendorf Histograph electrode system (11, 12). Extensive hypoxia has also been found in tumors of the prostate and breast (13, 14). Thus, tumor oxygenation is now recognized as a strong prognostic indicator. However, the Histograph is highly invasive and it is not possible make repeated measurements at individual locations, precluding dynamic studies to assess the influence of interventions on tumor pO_2 . A non-invasive imaging approach would be preferable.

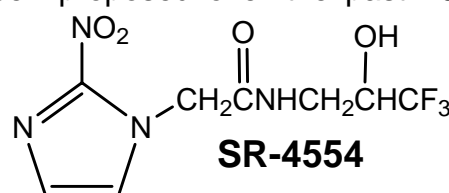
As an ultimate goal, oximetry would be based on endogenous tissue properties.

Hemoglobin is a candidate reporter molecule and near infrared spectroscopy can provide a direct measure of variation in tumor hematocrit (*viz.* blood volume) and relative hemoglobin oxygen saturation (15). Based on the paramagnetic property of deoxyhemoglobin BOLD (Blood Oxygen level Dependant) contrast ^1H MRI provides high spatial and temporal resolution related to hemoglobin oxygen saturation, but signal also responds to variations in vascular volume, and flow and measurements provide relative pO_2 rather than absolute values (16, 17). The BOLD effect is the foundation of fMRI used extensively in neurological research and we have successfully implemented it to assess response to adjuvant chemotherapy for advanced local breast cancer (18). However, changes in vascular oxygenation may not coincide with tissue pO_2 , which is a balance between oxygen delivery and consumption and clearance. Many biochemical pathways are under oxygen regulation and can provide an elegant window on hypoxia, *e.g.*, induction of HIF-1 and Glut-1 together with secondary responses such as increased production of VEGF, NIP3 and tumor associated macrophage activity (9). To date, such approaches have been limited to histology, requiring biopsy. Another approach is to adopt hypoxic response elements (HREs) as promoter sequences coupled to reporter genes, such as GFP (green fluorescent protein) or luciferase.

More generally, tumor oxygenation has been evaluated using specific exogenous reporter molecules and agents have been developed for use with nuclear imaging, optical imaging, ESR, and NMR. Two fundamental approaches evaluate either hypoxia or pO_2 .

Hypoxia

Specific classes of reporter molecule have been developed to reveal hypoxia (*e.g.*, pimonidazole, EF5, CCI-103F, Cu-ATSM, galactopyranoside IAZA) (5). Following IV infusion, these agents become reduced in tissues and are trapped. However, in the presence of oxygen they are reoxidized and ultimately clear from the body. Histological assessment of the distribution of these agents provides microscopic indications of local hypoxia. EF5, pimonidazole, and Cu-ATSM are currently being tested in clinical trials and correlations have been reported with clinical outcome. Many variants have been proposed over the past 20 years and incorporation of radionuclides has facilitated non-invasive investigations using PET or SPECT, while ^{19}F labels permit NMR spectroscopy (19). Several ^{19}F NMR hypoxia agents have been tested, *e.g.*, hexafluoromisonidazole (CCI-103F), EF5, NLQ-1, SR-4554, and Ro 07-0741). SR-4554 is being evaluated in an ongoing clinical trial (20).



Assessment of hypoxia is predicated on uptake and trapping, which are assessed as the relative signal at various time points (retention index) or based on the relative signals from tumor and surrounding control tissues. Weak signals generally restrict measurements to a global value across the whole tumor. Importantly, correlations between uptake in murine tumors and radiobiological hypoxic fraction have been reported, though intriguingly not with electrode measurement of pO_2 . It has been suggested that the latter mismatch arises from the relative contribution of chronic and acute hypoxia. Trapping may also depend on expression of nitroreductases and be modulated by glutathione. Likewise, tumor perfusion could influence access of the agents to tumor tissue, particularly poorly perfused regions, which are expected to be hypoxic. One might also expect the NMR signal to be broadened upon adduct formation with macromolecules (21). A typical dose has been reported as 180 mg/kg IP in mice (19) or 1400 mg/m² for patients (20). Generally, only a single time point is investigated, but dynamic variations in hypoxia may be assessed, even in biopsy specimens, by applying pairs of hypoxia

reporters in a pulse chase fashion (22). Direct detection of hypoxia is essentially a chemical approach whereby reporter molecules are reduced and trapped as non-specific adducts or reoxidized and cleared.

Oximetry

pO₂ may be measured directly using physical interactions between oxygen and reporter molecules. Phosphorescent and fluorescent agents have been used for optical measurements based on oxygen dependant signal quenching. Reporter molecules have been developed for use with ESR, where the linewidth is highly sensitive to oxygen (6, 7). NMR oximetry has generally followed the pioneering work of Thomas *et al.* (23), who showed that tissue pO₂ could be imaged in various organs based on the ¹⁹F NMR spin lattice relaxation rate ($R_1=1/T_1$) of perfluorocarbon reporter molecules following IV infusion of emulsion. At any given magnetic field (B₀) and temperature (T) sensitivity to changes in pO₂ is given by $R_1 = a + bpO_2$. This linear relaxation rate dependence arises from the paramagnetic oxygen, which is highly soluble in perfluorocarbons. According to Henry's Law, the dissolved mole fraction is related directly to the partial pressure of oxygen and $R_1 = R_{1a} + (R_{1p}/K)pO_2$. The slope (R_{1p}/K) indicates the response of a particular resonance to pO₂.

The sensitivity of R₁ of individual perfluorocarbon resonances varies widely and depends on the intrinsic anoxic relaxation rate, the solubility of oxygen and the ability of the oxygen molecule to approach molecular moieties. Another consideration is R₁ sensitivity to temperature. Over small temperature ranges, a linear correction to calibration curves is appropriate. However, it is preferable for a pO₂ sensor to exhibit minimal response to temperature, since this is not always known precisely *in vivo* and temperature gradients may occur across tumors. Even a relatively small error in temperature estimate can introduce a sizable discrepancy into the apparent pO₂, e.g., the relative error introduced into a pO₂ determination by a 1 °C error in temperature estimate ranges from 8 torr/°C for perfluorotributylamine, to 3 torr/°C for PFOB (perflubron) or 15-crown-5-ether (15C5) (24) and 0.1 torr/°C for hexafluorobenzene (HFB) (1), when pO₂ is actually 5 torr.

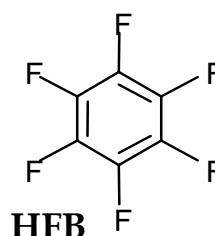
PFCs essentially act as molecular amplifiers, since the solubility of oxygen is greater than in water, but thermodynamics require that the pO₂ in the PFC will rapidly equilibrate with the surrounding medium, and estimates of diffusion suggest the equilibration occurs within seconds. Since relaxation is proportional to oxygen concentration, the effect is greater at a given pO₂ than for water. Importantly, ions do not enter the hydrophobic PFC phase, and thus, do not affect the bulk relaxation. Indeed, PFCs are typically exceedingly hydrophobic and do not mix with the aqueous phases, but rather form droplets or emulsions. Based on these principles, PFCs have been applied to *in vivo* pO₂ measurements.

The most popular route for the delivery of PFCs is as emulsions injected intravenously. Given the extremely hydrophobic nature of PFCs, they do not dissolve in blood directly, but may be formulated as biocompatible emulsions. Some investigators have undertaken MR spectroscopy and imaging relaxometry of PFC in the blood, providing measurements of vascular pO₂. Primary vascular clearance is by macrophage activity over 1 to 2 days leading to extensive accumulation in the liver, spleen, and bone marrow. Indeed, this is a major shortcoming of IV delivery, since animals may exhibit extensive hepatomegaly. Many investigators have measured tissue pO₂ in liver, spleen, and tumors following clearance from the blood (1, 23-25). Typically, 100s µl of emulsion are required with mice and several ml for studies in rats.

Many PFCs (e.g., perfluorotributylamine (PFTB), perflubron PFOB), Therox™ (F44-E)) have several ^{19}F NMR resonances, which can be exploited to provide additional information in spectroscopic studies, but seriously hamper effective imaging (25). Multiple resonances can lead to chemical shift artifacts in images, which compromise the integrity of relaxation rate measurements, though they can be avoided by selective excitation, or detection, chemical shift imaging, deconvolution or sophisticated tricks of NMR spin physics. Thus, a PFC exhibiting a single ^{19}F NMR resonance is preferable. Choice of PFC is also governed by practical considerations, such as cost and availability, since several products, particularly, proprietary emulsions may be difficult to obtain.

Both spectroscopic and imaging approaches have been applied to tissue pO_2 measurements depending on the available signal-to-noise. It appears that uptake and distribution efficiency following IV administration vary with tumor type, but in general, maximum signal is detected from the tumor periphery corresponding to regions of greater perfusion (26). Several reports have examined changes in tumor pO_2 in response to acute interventions, such as vasoactive drugs and hyperoxic gases. Spectroscopic time resolution has ranged from seconds to minutes, while imaging often takes longer. Long tissue retention facilitates chronic studies during tumor development and progressive tumor hypoxiation has been observed over extended periods of many days (26, 27). To avoid reticuloendothelial uptake and bias towards well perfused regions, we favor direct intratumoral (IT) injection of neat PFC allowing any region of interest in a tumor to be interrogated immediately. Use of a fine needle ensures minimal tissue damage.

Prompted by earlier studies, we surveyed a number of commercial PFCs and identified that hexafluorobenzene (HFB) has many virtues as a pO_2 reporter (28). Symmetry provides a single narrow ^{19}F NMR signal and the spin lattice relaxation rate is highly sensitive to changes in pO_2 , yet minimally responsive to temperature. HFB is cheap, readily available commercially in high purity, and well characterized in terms of lack of toxicity (29). Following initial spectroscopic studies, which used 10-20 μl HFB injected into the tumor center or periphery, we have now developed rapid imaging methods (1). Recognizing that tumors are heterogeneous and that pO_2 may fluctuate, we developed a procedure [*FREDO* (Fluorocarbon Relaxometry using Echo planar imaging for Dynamic Oxygen Mapping)], which allows repeated quantitative maps of regional pO_2 to be achieved with multiple individual locations (50-150) simultaneously in 6.5 mins with a precision of 1-3 torr, when pO_2 is in the range 0-15 torr (1). HFB is highly volatile and does not form stable emulsions, however, the mobile fluid allows a very fine sharp needle (32 Gauge) to be used for intra tumoral injection at multiple locations. Overlaying ^{19}F MR images on the corresponding proton images reveals the distribution of HFB. Typically, 50 μl HFB are injected and we typically interrogate 5 – 10 % of a tumor. Highly consistent inter tumor behavior between multiple tumors of a given type (and size) suggests appropriate sampling. Since HFB is highly volatile and clears from tumors within 24 h, repeated measurements on subsequent days for chronic longitudinal investigations usually requires re-administration of the HFB: the highly consistent data achieved in tumors with such successive measurements indicates the effective representation of the true distribution of oxygen tensions within the tumors (30).



At 37 °C and 4.7 T: pO_2 (torr) = $(R1(\text{s}^{-1}) - 0.0835)/0.001876$, so that T_1 reaches 12 s under anoxic conditions. To avoid excessive experimental time we favor pulse burst saturation

recovery (PBSR) echo planar imaging (EPI) relaxometry. To further enhance measurements, we apply the ARDVARC (Alternated Relaxation Delays with Variable Acquisitions to Reduce Clearance effects) acquisition protocol (1). Traditional T1 measurement sequences acquire data with delays in monotonic order, whereas we alternate longer and shorter delays to minimize any systematic errors, which would be introduced, if the signal amplitude varies during the measurement. The most powerful aspect of *FREDOM* is the ability to follow the fate of individual voxels with respect to interventions. We usually acquire at least three baseline pO₂ maps followed by further maps accompanying interventions, such as hyperoxic gas breathing. Even under baseline conditions, fluctuations in T1 are apparent. These may arise from uncertainty in T1, which may be reflected in T1_{err} or transient fluctuations in pO₂. As with any measurement, sampling is a critical issue. *FREDOM* is analogous to the Eppendorf Histogram, in that it samples multiple locations, which appear to reflect interstitial pO₂. Comparison of pO₂ distributions using *FREDOM* or the Eppendorf Histogram has shown close similarity in both small and large tumors (31). However, *FREDOM* has the tremendous advantage over the Histogram of permitting dynamic measurements with respect to interventions. Dynamic studies in several tumor types have shown equivalent behavior when assessed using polarographic oxygen electrodes or OxyLite™ or FOXY™ optical probes (30, 32). Relative hypoxia has been compared with the histological reporter pimonidazole revealing similar trends across tumor types (33). Data may be presented as histograms revealing significant differences between mean and median pO₂ and hypoxic fractions between small and large tumors and between the slow and fast growing sublines H and AT1 of the Dunning prostate R3327. *FREDOM* has been applied to investigations of diverse tumor types (syngeneic rat prostate and breast tumors and xenograft human lymphomas) with respect to growth and acute interventions. Zhao *et al.* (34) recently demonstrated rapid hypoxiation of rat breast tumors following administration of the vascular targeting agent Combretastatin with differential localized recovery 24 h later. Most significantly, it has been shown that the ability to modulate pO₂, as assessed using *FREDOM* correlated with tumor growth delay accompanying irradiation (35). In some tumor types, there is a strong correlation between mean pO₂ and hypoxic fraction, though this is not always the case.

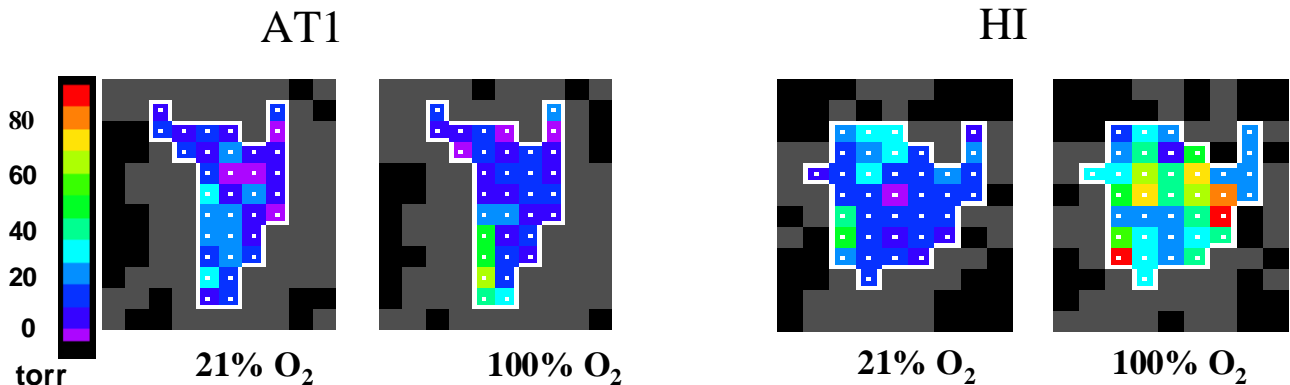


Fig. 1 PO₂ maps obtained from Dunning prostate R3327-AT1 and HI tumors when anesthetized rats breathed air and then oxygen. Under baseline conditions, the hypoxic fractions appear quite similar, but there is a markedly different response to breathing hyperoxic gas. In the AT1 tumor, only those regions, which were initially well oxygenated, responded. By contrast, in the HI all tumor regions, irrespective of baseline pO₂ increased dramatically (data kindly provided by Dr. Dawen Zhao).

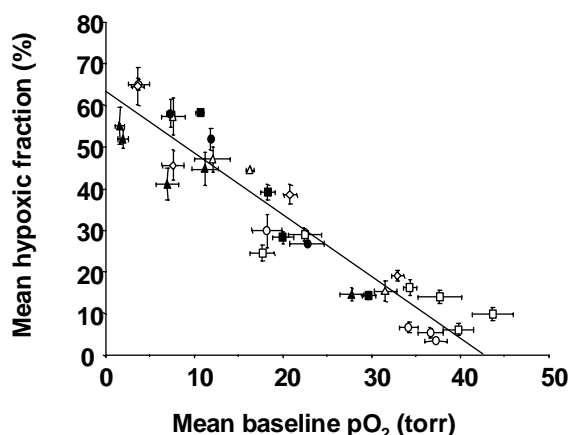
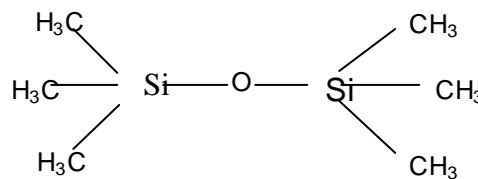


Fig. 2 Correlation between baseline hypoxic fraction (HF₁₀; mean \pm SE) and mean baseline pO₂ for a group of 7 Dunning R3327-HI tumors assessed repeatedly at various sizes showing the expected inverse relationship ($R > 0.9$) (modified from (30)).

For small animal work, ¹⁹F NMR is widely available at 4.7, 7 and 9.4 T by minor adaptation of routine instrumentation, e.g., retuning proton RF coils. Within the recent past ¹⁹F MRI is also becoming available on clinical systems, facilitating translation of these techniques to patients. ¹⁹F NMR is particularly facile because there is essentially no background signal in tissues to interfere with measurements, yet the resonance frequency and sensitivity approach that of proton NMR. Proton NMR studies have shown changes in the tissue water relaxation rate with varying oxygenation, but many other processes (metal ions, cellularity, pH, ionic strength) also cause relaxation and the relaxivity due to oxygen is small. However, proton MRI is routinely applied for anatomical evaluation of tumors and would provide an ideal conduit for prognostic investigations. We have recently found a proton analog of HFB, specifically hexamethyldisiloxane (HMDSO). Like HFB, HMDSO is highly hydrophobic giving high gas solubility, and hence, strong R1 response to changes in pO₂. Symmetry provides a single proton resonance ($\delta = 0$ ppm), which is well removed from water and fat. Preliminary data suggest it provides a viable new approach to tumor oximetry (36).



Each technique has specific virtues and drawbacks, which must be considered for any given application. In particular, the degree of invasiveness, the ability to generate maps of heterogeneity and the ability to assess dynamic changes. In addition, the location of a measurement, e.g., vascular vs. tissue compartments, the precision of measurements and spatial and temporal resolution. For further details of the techniques described above, the reader is referred to the references.

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